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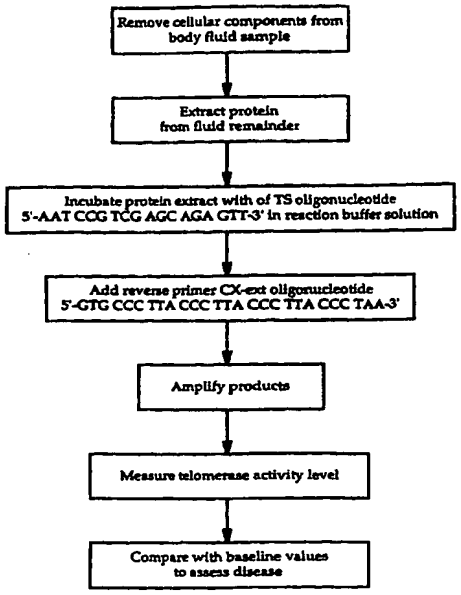
(54) Title: TELOMERASE ASSAY OF BODY FLUIDS FOR CANCER SCREENING AND ASSESSMENT OF DISEASE STAGE AND PROGNOSIS

(57) Abstract

Elevated telomerase activity levels in tissue and cell extracts have been used to determine the presence of cancer in a tissue sample. The invention herein is directed to an improved telomeric repeat amplification protocol (TRAP) assay for quantitating telomerase RNA or telomerase activity levels, using easily obtainable body fluids, such as blood, plasma, lymph, pleural fluid, spinal fluid, saliva, sputum, urine, and semen, for example, to both detect the presence of cancer as well as assess the stage of the disease and the prognosis of the patient. The improved TRAP assay can be used as a diagnostic marker for malignancy as well as a means of monitoring the progress and effectiveness of cancer therapies.



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TITLE OF THE INVENTION

TELOMERASE ASSAY OF BODY FLUIDS FOR CANCER SCREENING AND ASSESSMENT OF DISEASE STAGE AND PROGNOSIS

The development of the present invention was supported by the University of Maryland, Baltimore.

FIELD OF THE INVENTION

The invention is in the field of minimally invasive cancer detection and assessment. The telomerase assay disclosed and claimed herein has both diagnostic and therapeutic utility.

BACKGROUND OF THE INVENTION

In 1972, James Watson described what is now known as the end-replication problem, the inability of DNA polymerase to fully replicate the ends of a linear DNA molecule **(Figure 1)**. The end replication problem predicts the progressive reduction of chromosomal DNA at the 5 'end of the newly replicated daughter strand during multiple cell cycles. The loss of genomic sequences at each replication cycle can be compensated for by addition of terminal sequences by an enzymatic reaction involving terminal transferase or "telomerase", a ribonucleoprotein complex that utilizes sequences of its own RNA component as a template for the de novo synthesis of telomeric DNA sequences.

Most somatic cells lose telomere DNA steadily as they divide and do not possess detectable telomerase activity [Kim et al., *Science*, 266: 2011-2014 (1994)].

Because telomerase prevents degradation of the chromosomal DNA, it has essential functions for cell immortalization. The current view of carcinogenesis is that uncontrolled cell proliferation is part of the development of malignant disease and uncontrolled cell populations that do progress to the malignant state attain the ability to replicate indefinitely. Clearly, the enzyme telomerase is vital for unlimited cell proliferation. Thus, with few exceptions, telomerase is highly expressed in and specific to malignant cells. It has been found in approximately 90% of all tumor tissues tested (n=2000), making telomerase a universal tumor marker [Shay et al., *Trends Genet* , 12: 129-131 (1996)].

The majority of deaths from cancer result from metastases that are resistant to conventional therapies. Despite improvement in diagnosis, surgical techniques, and general patient care many cancers can not be eradicated because by the time the initial diagnosis is made, metastasis may have already occurred [Fidler et al., *Cancer Res*, 50:6130-6134 (1990)]. The ability to detect cancer early may improve cure rates by allowing a tumor to be treated when it is smaller, less aggressive, and less likely to have metastasized. In contrast to the short time between disease presentation and established metastasis, the chemoprevention or diagnosis period may extend back 10 years or more [Kelloff et al., *Cancer Res*, 54:2015-2024 (1994); Mulshine et al., *Chest*, 55:280S-286S (1994)]. Tumor growth is characterized by distinct phases, from dysplasia to carcinoma to angiogenesis, that present long before the tumor can be detected by known means **(Figure 2)**. On average, a tumor will double in cell number every 3 months [Israel, *J Theor Biol*, 178: 375-380 (1996)]. At approximately 20 doublings, which is equivalent to a tumor of one million cells, the tumor undergoes angiogenesis, thus acquiring a

metastatic potential. The acquisition of an adequate blood supply (i.e. angiogenesis) is required in order for the tumor mass to exceed 2 mm in diameter [Folkman, *Nature Med*, 1: 27-33 (1995)]. Today's technology rarely enables us to detect tumors smaller than one billion cells (depending on the specific disease type and stage), which is equivalent to approximately 30 doublings. Typically, a patient will present with considerably more tumor burden than that [Mulshine et al., *Oncology*, 5: 25-32 (1991)]. However, even 30 doublings is very late in the natural history of tumor growth. Frequently, metastases have already occurred, and the patients will eventually die of their disease [Nicolson, *Bioessays*, 13: 337-342 (1991)].

It has long been known that tumor cells have a life cycle. Although malignant tumors are "immortal", cells within tumors become necrotic due to lack of oxygen and nutrients and are then shed into the bloodstream at a constant log rate. After angiogenesis occurs, cells on the exterior of tumors are also constantly shed into the bloodstream and this release of cells increases in rapidly growing tumors [Kohn et al., *Cancer Res*, 55: 1856-1861 (1995)]. While not wishing to be bound by theory, we believe that, due to the natural life cycle of tumor cells shedding into the bloodstream, evidence of malignant cells can be detected in circulation as early as 20 to 30 doublings of the life cycle of the tumor. Such, a detection method sensitive enough to measure circulating malignant cells would be better than all clinical detection methods used today.

To date, accurate and early detection of cancer by analysis of tumor markers in circulation has not been achieved due to several reasons: (a) the tumor marker levels may be too low to be detected in the circulation by known

methods; (b) the tumor marker may not be specific to malignant cells, resulting in a high false positive rate; (c) the tumor marker may be found in only a percentage of the tumors tested, resulting in a high false negative rate [Canney et al., *Br J Cancer*, 50: 765-769 (1984); Bates et al., *Cancer Treat Rev*, 12: 163-207(1985); Scott et al., *J Cell Biochem*, 17: 175-183 (1993)]. The development of a non-invasive assay that quantitatively detects a tumor marker specific to all malignancies leading to early diagnosis of cancer would represent a major breakthrough in the field of cancer detection.

Many researchers have correlated telomerase activity in tumor tissues with prognosis in various cancers such as neuroblastoma, acute myeloid leukemia, breast, and gastrointestinal cancers [Hiyama et al., *Nature Med*, 1: 249-255 (1995); Counter et al., *Blood*, 85: 2315-2320 (1995); Zhang et al., *Clin Cancer Res*, 2: 799-803 (1996); Hiyama et al., *J Natl Cancer Inst*, 88 (1996); Tahara et al., *Semin Oncol*, 23: 307-315 (1995)]. All of these studies have been carried out on the tumors themselves. However, in order for measurements of a tumor marker such as telomerase to be useful in screening for cancer, one must address several critical questions including: is the marker differentially expressed in normal and high-risk tissue; at what stage of progression does the marker appear; is there a correlation with clinical outcome; what are the acceptable specificities, sensitivities and accuracies of the assay; and can the assay distinguish between aggressive cancers that will progress from those that will not? Data from previous studies have shown that telomerase activity is a valid marker in the diagnosis and prognosis of cancer [Hiyama et al., (1995); Shay et al., (1996); Hiyama et al., (1996); Tahara et al., (1996)].

To date, all studies involving telomerase activity in cancer have been done using samples directly from the *tumor itself*. For example, detection of telomerase activity in lung cancer patients is done by examining biopsied or surgically resected lung tumor; in brain tumor patients, by examining the brain tumor, etc. In the case of blood tumors (leukemia), a sample of white blood cells is examined.

Our invention is fundamentally different and superior. We have discovered that it is possible to detect evidence of malignant cells shed by organs other than blood in the *non-cellular portion of the blood* of cancer patients. For example, we have detected elevated telomerase activity levlels in the non-cellular portion of the blood of lung cancer patients whose tumor is not a blood tumor. We utilize a newly configured telomeric repeat amplification protocol (or TRAP) assay. Using this modified TRAP assay, we can develop a blood test screen for cancer.

In addition to the clinical utilities in cancer diagnosis and prognosis, the level of telomerase expression can be used to monitor cancer therapy effectiveness. The failure of telomerase activity to regress to within the normal barely detectable range in blood after chemotherapy will be predictive of persistent disease. In such cases, therapy can be stopped or changed in an effort to limit spread of the disease. Baseline values of telomerase activity derived from a control population of healthy individuals will indicate successful therapy and provide an end point to treatment. Using easily obtainable body fluid, telomerase activity levels can be measured to determine the most effective therapy regimens for patients and aid in reducing cancer recurrences. Patients in

remission can be monitored for telomerase activity levels in blood during follow-up visits. An increase in telomerase activity will indicate relapse earlier when treatment is more effective.

Genetic changes that lead to an immortalized cell may be its ultimate undoing. In order for a malignant cell to proliferate infinitely, its telomere ends must be maintained at a stable length [Harley, *Mutat Res*, 256: 271-263 (1991)]. In ~90% of malignant tumors, chromosome end integrity is maintained by telomerase [Shay et al., (1996)]. Using the techniques disclosed herein, a minuscule blood sample can be analyzed for telomerase levels and reveal the presence of a new or metastatic tumor, be it of the lung, liver, breast or any organ, in time to eradicate it. The sensitivity of this non-invasive test could change our conception of cancer. Rather than becoming a diagnosis leading to limited treatment options, early stage tumors will be caught and eradicated.

SUMMARY OF THE INVENTION

It is the object of the invention to provide a protocol to determine the presence of telomerase and quantitate levels of telomerase activity in the body fluid of cancer patients.

It is a further object of this invention to use the telomerase and telomerase activity measurements as a tumor marker for diagnostic purposes. It is a further object of the invention to use these telomerase and telomerase activity measurements to monitor in a minimally invasive manner the status of cancer patients and assess prognosis of the disease.

It is a further object of the invention to develop a cancer screening kit for routine use on healthy individuals to detect cancer in the early stages using easily obtainable body fluids. It is a further object of the invention to correlate measured telomerase and telomerase activity with the tumor bulk and grading and/or staging of the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: The end-replication problem (prior art)

Figure 2: Time windows for intervention *vs.* treatment. (prior art)

Figure 3: Telomerase repeat amplification protocol (TRAP) assay of body fluids.

Figure 4: Plasma telomerase activity (expressed as a percentage of the activity in 50 K562 cells) in control.

Figure 5: Plasma telomerase activity in cancer patients.

Figure 6: Plasma telomerase activity in cancer patients in remission versus those with aggressive lung cancer.

DETAILED DESCRIPTION OF THE INVENTION

To study the instant invention, we chose lung cancer patients as the primary study because the lungs contain a very dense capillary bed and receive the complete cardiac output every minute [West, *Wilkins and Wilkins* (1982)]. Therefore, lung tumors have easy access to the body's circulation system which increases the likelihood of detecting a tumor marker in blood. In general, lung cancers are separated by histology into two broad subtypes: 1) small cell lung cancer (SCLC), and 2) non-small cell lung cancer (NSCLC). This classification is widely accepted and necessary due to major differences in therapeutic approach in patients with SCLC versus NSCLC [Hirsch et al., *Cancer*, 62: 973-980 (1988)]. For our study, NSCLC includes all lung cancers without a small cell component including: (a) adenocarcinoma, (b) squamous cell carcinoma, and (c) large cell carcinoma.

Lung cancer is one of the most common fatal malignancies in the world, accounting for more than 28% of all cancer deaths each year [Miller et al., *Natl Cancer Inst*, 93: 2789-2795 (1993)]. Despite continuing research into new therapeutic strategies, overall lung cancer survival has not improved during the past two decades, with 5-year survival remaining about 13% [Ries et al., *Natl Cancer Inst*, 94: 263-276 (1994)]. Although the 5-year survival for metastatic lung cancer remains under 1.5%, the overall and disease free survival of early stage lung cancer is encouraging, making the prospect of early diagnosis quite beneficial. The 5-year survival for stage 1 lung cancer in which the tumor is relatively small and there is no nodal involvement has been reported as high as 80% [Ries et al., (1994)]. However, most lung cancer deaths are due to distant

metastatic disease. Typically, when a patient presents with initial complaints or for isolated screening, only very big lesions (those that measure 3 or 4 to 7 cm in diameter) are easily visible by current diagnostic procedures such as chest radiography [Mulshine et al., (1994)]. These lesions represent advanced cancers that are usually metastatic. Currently, there are no screening procedures for detection of lung cancer early enough to increase long term survivability [Rimer et al., *Handbook of Health behavior research,* (1997)].

In order for a tumor marker such as telomerase to be utilized in the assessment of malignancy in blood samples, it must be expressed by the primary tumor. Three studies have been published regarding telomerase activity in lung cancer tumors [Kim et al., (1994); Hiyama et al., *J Natl Cancer Inst,* 87: 895-902 (1995); Shirotani et al., *Lung Cancer,* 11: 29-41 (1994)]. Pooling the results from these studies, telomerase activity was detected in 78% (n=125) of NSCLC tumors and 100% (n=15) of SCLC tumors. Highest telomerase activity was detected in SCLC which displayed levels comparable to immortalized lung cancer cell lines used as positive controls [Hiyama et al.,*J Natl Cancer Inst* (1995)]. Small cell lung cancer is a very aggressive malignancy that progresses quickly to widespread disease. At presentation, more than 90% of SCLC patients have advanced and/or metastatic disease [Feld et al., *Chest,* 103: 1010-1015 (1996)]. Therefore, extremely high levels of telomerase in SCLC tumors is not surprising given the correlation of high telomerase activity in advanced stage cancers [Shay, (1996)].

In addition to detecting malignant disease in its early stages, quantitative measurements of telomerase activity in blood can provide prognostic data. After the initial diagnosis of lung cancer, tumor volume is assessed by a clinical staging

evaluation. This evaluation confirms histologic data, determines whether metastasis is present, and assesses the patient for operability. If the tumor is relatively confined-stage 1 or 2-surgical resection is generally recommended. However, after surgery as much as 35% of patients are found to be understaged [Ihde, *Curr Prob Cancer*, 15: 65-72 (1991)]. In these patients surgical removal of the tumor does not improve prognosis and is thus an unnecessary invasive procedure. Measurements of telomerase activity levels, however, can provide data helpful in the accurate clinical staging of cancer. Tumor marker levels are strongly related to tumor bulk, being relatively low when disease spread is limited [Crippa et al.,*J Nucl Biol Med*, 36: 52-55 (1992)]. Therefore, very high telomerase activity levels correlates to patients with extensive disease who are not good surgical candidates.

The ascertainment of how quickly a tumor will grow or spread is a critical component in determining post-surgical treatment, either radiation or chemotherapy. In NSCLC, patients whose tumors have a low proportion of cells in G0 or G1 phase have shorter survival times [Volm et al., *Cancer*, 56: 1396-1401 (1985)]. Since telomerase is down-regulated in the G0 phase, tumors with high telomerase activity will generally contain a low number of cells in G0 [Holt et al., *Mol Cellular Biol*, 16(6): 2932-2939 (1996)]. Therefore, measurements of telomerase activity in primary tumors can distinguish rapidly growing tumors from indolent disease. One can use these telomerase measurements o develop specific therapies based on aggressiveness of the cancer.

The detection of occult neoplastic cells in surgical resection margins is a strong predictor of local regional recurrence resulting in a significant decrease in

overall survival [Bartlett et al., *Germ Cell Neoplasm*, 5(6): 1245-1260 (1991)]. In head and neck cancer, for example, the tumors are molecular typed for p53 mutations. The tumor is then a searched for these same mutations in resection margins to identify patients with residual tumor cells undiscovered by standard pathological examination [Brennan et al., *N Engl J Med*, 332: 429-435 (1995)]. These patients appeared to be at high risk for relapse and their identification allowed for the addition of local therapy such as radiation. The same approach, using telomerase activity as a marker, can be applied to tumor resection margins in lung cancer. Telomerase activity can be detected in tissues composed of only .01% telomerase positive cells [Wright et al., *Trends Cell Biol*, 5: 293-297 (1995)]. Current histological techniques, on the other hand, may fail to detect metastasis in sections containing less than 2% neoplastic cells [Brennan et al.,(1995)]. In addition, quantitative measurements of telomerase activity in serial blood samples taken from the same patient may reveal evidence of residual disease outside of tumor resection margins. After tumor resection, telomerase activity should decrease at its theoretic half life in blood to a normal baseline value. A prolonged half life time may indicate the presence of residual or occult disease [Lange et al., *J Urol*, 128: 708-711 (1982)]. In breast cancer patients without evidence of disease and increasing tumor marker levels, preliminary data showed that administration of chemotherapy improved survival rate [Jager, *Eur J Cancer Prev*, 2(3): 133-139 (1993)]. Therefore, measurements of telomerase activity in tumor resection margins and blood can provide a sensitive means to assess radicality of surgery for primary tumor.

Blood Sample Collection And Preparation

The following variables were tested for their effect on measurable telomerase activity in blood samples: 1) type of blood drawing tube (heparinized, EDTA, sodium citrate, or no additives) 2) time elapsed between blood draw and protein extraction (0, 2, 4, and 24 hours), 3) temperature at which sample was held until protein extraction (room temperature vs. refrigerated), and 4) addition of RNase inhibitors to protein lysis buffer. Each blood sample was "spiked" with K562 cell extract, subjected to the aforementioned variables, and assayed for telomerase activity. K562 is a human leukemia cell line that has been shown to exhibit very high telomerase activity [Kim (1994)]. We used a protein extract of this cell line as a positive control in the development of our assay. Each blood sample was centrifuged for 5 minutes at 125 x g at room temperature to pellet the cellular fraction. One hundred microliters of plasma from each sample was added to one milliliter of a lysis buffer containing the following: 0.5% CHAPS, 10 mM TRIS-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 5 mM beta-mercaptoethanol, 0.2 mM AEBSF, and 80 units of broad range RNase inhibitor (obtained from Promega). The volume was centrifuged at 17,000 g for 20 minutes at 4 degrees celsius. Two-thirds of the supernatant are collected and the protein concentration determined as described below. Two microliters of protein extract (containing 9 and 12 micrograms protein) were assayed for telomerase activity. Blood samples drawn in sodium heparin vacutainers, held no longer than 4 hours at room temperature best preserved telomerase activity.

Measurement of Telomerase Activity

Telomerase activity in cells is standardly measured using the TRAP (telomeric repeat amplification protocol) assay developed by Kim and Shay. The TRAP assay measures telomerase activity in protein extracts isolated directly from tumor tissues and tumor cells [Kim et al., (1994)]. However, telomerase has not been measured in blood fluids for two reasons. First, telomerase is a cellular enzyme, found only in cell nuclei, and therefore was not expected to be detectable in non-cellular tissue components such as blood fluid or plasma. Second, detecting telomerase activity in tissues containing blood components by the known TRAP assay would be expected to present a technical challenge for the following reasons: (1) heme products found in blood have a major inhibitory effect on the PCR amplification, an integral part of the TRAP assay; and (2) ribonucleases (RNases), which are released in the blood by the destruction of lysosomes, should inactivate telomerase by degrading its ribonucleic acid (RNA) template, rendering telomerase activity undetectable by the TRAP assay [Akane et al. , *J Forensic Sci*, 39: 362-372 (1994)]. Given these drawbacks, the likelihood of detecting telomerase activity or developing an assay to quantitate telomerase activity in a non-cellular, RNase containing fluid such as plasma seemed remote to those skilled in the art.

Unexpectedly, we found that telomerase activity could indeed be measured in serum such as blood or plasma. We modified the known TRAP assay so as to achieve increased sensitivity of telomerase detection in dilute protein extracts as well as more uniform results between duplicate assays. Our assay is performed using different PCR tubes and the reaction buffer has been modified. The timing,

temperature, and even the reverse primer have been changed. The new reverse primer limits staggered annealing of the primers, which virtually eliminates false positive results caused by primer/dimer artifacts. A high fidelity Taq polymerase replaces the recommended Taq polymerase from Gibco, BRL. These improvements have increased the sensitivity of the telomerase activity detection as well as improved the reliability of the assay.

Specifically, we extracted protein from collected blood samples of patient subjects. The protein extract was incubated for 45 minutes at 30 degrees C with 0.250 ug of TS oligonucleotide (5'-AAT CCG TCG AGC AGA GTT-3') (SEQ ID NO. 1) in thin-walled reaction tubes (obtained from Perkin Elmer) that contained 20 mM TRIS-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20 (Sigma), 1 mM EGTA, 25 uM each dNTP, 0.5 uM T4 gene 32 protein (obtained from Ambion), and 2.5 Ci -^{32}P dCTP (3000 Ci/mmol) in a total volume of 25 l. During the 45 minute incubation, telomerase adds TTAGGG repeats to the TS oligonucleotide. The PCR tubes were heated to 94 degrees C for 2 minutes to inactivate telomerase and decrease formation of primer/dimers. Next, 25 microliters of reaction buffer, heated to 94% C, containing 20 mM TRIS-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20 (obtained from Sigma), 1 mM EGTA, 25 uM each dNTP, 2.5 U Hi Fi Taq polymerase (obtained from Boehringer-Mannheim), 0.1 micrograms CX-ext oligonucleotide (5'-GTG CCC TTA CCC TTA CCC TTA CCC TAA-3') (SEQ ID NO. 2), and 5.0 attg ITAS (internal telomerase assay standard) were added to bring the volume to 50 microliters. The ITAS is a 150 base pair DNA fragment with binding sites for the forward TS

primer and the reverse CX primer. The products were amplified for 30 cycles at 94 degrees C for 30 seconds, 50 degrees C for 30 seconds, and 72 degrees C for 45 seconds. A 10 microliter aliquot of the amplified products were resolved on a 12.5% non-denaturing polyacrylamide gel in 45 mM TRIS base/45 mM boric acid/1 mM EDTA at 200 volts for 30 minutes, followed by 2.5 hours at 275 volts. The gels were dried and exposed for 16 hours to PhosphorImager screens (Molecular Dynamics). Positive and negative controls were run in parallel with each experiment.

Quantitation of the TRAP assay

To determine the conditions under which our TRAP assay operated in the linear range, reactions were performed with serial dilutions of the K562 cell and tissue protein extracts. In all cases 0.06 ug protein extract was chosen to perform the assay. The TRAP reactions were quantitated using ImageQuant software on a Molecular Dynamics PhosphorImager. The signal intensities of each TRAP product band were measured and corrected for background by subtracting the signal intensity measurement of the TRAP reaction without protein extract. The samples were given arbitrary units of activity by summing the adjusted signal intensities of the TRAP bands. The level of specific telomerase activity in 50 K562 cells was set to 100% in each assay. The specific telomerase activity of each sample was expressed as a percentage of the telomerase activity in 50 K562 cells, rounded to the nearest percent.

Measurement of Telomerase by RNA PCR Analysis

Total RNA was isolated from 140 µl of plasma protein (preparation of plasma protein lysate done according to the protocol listed above) using the QIAamp viral RNA isolation kit (QIAGEN). The RNA is stored at 70 °C. Analysis of the expression of the telomerase RNA template (hTR) was performed by RT-PCR. hTR mRNA was amplified using the primer pair 5'- TCT AAC CCT AAC TGA GAA GGG CGT AG-3' (F3B) (SEQ ID NO. 3) AND 5'-GTT GCT CTA GAA TGA ACG GTG GAA G-3' (R3C) (SEQ ID NO. 4) as described by Feng et al. [*Science*, **269**: 1236-1241 (1995)].

First, 5 µl of total RNA are pipetted into thin walled 0.2 ml PCT tubes (Perkin-elmer, cat#N801-0737). The RNA is incubated at 94 °C for 4 minutes and placed immediately on ice. The incubation temperature allows for the elimination of RNA secondary structure, thereby allowing RNA to be reverse transcribed into cDNA.

Next, 45 µl of master mix (see Table 1) is added to each reaction tube on ice. The tubes are transferred to the PCR block and thermocycled using the hTR PCR program described below. The program includes the following incubation steps, wherein steps 3 to 5 are repeated 34 times:

(1) 42 °C for 45 minutes;

(2) 94 °C for 3 minutes, 30 seconds;

(3) 94 °C for 30 seconds;

(4) 57 °C for 30 seconds;

(5) 72 °C for 45 seconds;

(6) 72 °C for 2 minutes; and

(7) 4 °C hold.

COMPONENT	CONCENTRATION	10 ASSAYS (µl)	SOURCE, CAT#
10X TAQ BUFFER	1X	50	Perkin-Elmer Amplitaq
10 mM dNTPs	0.3 mM	15	Perkin-Elmer
25 mM MgCl	2.5 mM	50	Boehringer Mannheim
30 pM F3b	60 pM	20	Gibco-BRL, synthesized
30 pM R3c	60 pM	20	Gibco-BRL, synthesized
AMV RT	10 U	10	Promega
RNase inhibitor	20 U	5	Promega
Taq Pol	20 U	5.7	Boehringer Mannheim
DEPC water	-	274.3	Quality Biological

PCR products are analyzed by agarose gel electrophoresis. First, 5 µl of 10X loading buffer is added to each reaction tube. Next, 20 µl of PCR product is loaded on a 2% Nusieve gel (FMC Bioproducts) stained with 5 µl Ethidium Bromide (5mg/ml). A lane with a 25 base pair ladder (1 µg) is included. Products are electrophoresed at 125 volts for 45 minutes in 0.5X TBE. Gel is placed on the transilluminator and photographed. The expected PCR product is 126 base pairs.

The following examples are provided for illustrative purposes only, and are in no way intended to limit the scope of the present invention.

Example 1 -- Telomerase activity in a control population:

Blood samples were collected from 20 control subjects and tested repeatedly utilizing the modified TRAP assay disclosed herein. In all cases, telomerase activity was less than 5% of the positive control cell line **(Figure 4).**

Example 2 -- Telomerase activity in a cancer population:

Blood samples were collected from 20 lung cancer patients and tested in a double blind fashion utilizing the modified TRAP assay disclosed herein. Specifically, we were not informed of the patients disease status until after the tests; likewise, the clinician caring for the patient was not told of our results until after the patient was staged and prognosed, after the completion of the pilot study. In 14 cases, telomerase activity was less than or equal to 5% of the positive control cell line. In these 14 cases, it was subsequently learned that the patient was in complete or near complete remission at the time of testing. In the 6 cases with high telomerase activity (24% to 160% of the positive control), all had actively advancing and/or metastatic disease **(Figure 5)**. We plotted the plasma telomerase activity in the cancer patients as a function of disease prognosis, i.e. in complete or partial remission versus aggressive and/or metastatic disease **(Figure 6)**. In 100% of the patients in remission, telomerase activity levels were below 5%. In all but one patients, the telomerase activity levels were well above 5%. Because not every tumor tissue expresses elevated telomerase levels [found in

about 90% of tumor tissues tested, note Shay et al., (1996)], we would expect that an occasional patient would have a telomerase negative tumor.

Example 3 -- Telomerase activity as a means to assess cancer type and prognosis:

Blood samples were collected from 20 lung cancer patients and tested utilizing the modified TRAP assay disclosed herein. In 14 case cases, telomerase activity was less than or equal to 5% of the control cell line. In these 14 cases, the patient was in complete or near complete remission at the time of testing. In the 6 cases with high telomerase activity (24% to 160%), all had actively advancing and/or metastatic disease **(Figure 5)**.

All references cited herein are incorporated by reference in their entirety.

While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one with ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. For example, although the specific examples refer to testing the blood from *lung* cancer patients, it is clear that telomerase, being highly expressed in over 90% of tumor tissues, can be a diagnostic marker for other types of cancer. Second, although the telomerase assay disclosed herein is specifically discussed for testing protein extracts from *blood fluid*, it is clear that other body fluids such as lymph, pleural fluid, spinal fluid, saliva, sputum, urine, and semen, for example. Naturally, the particular body fluid selected for telomerase detection would depend on the patient, the type of cancer to be screened or assessed as well as the manifestation of the

disease. For example, it is clear that semen would not be the body fluid tested to diagnose ovarian cancer. By the same token, pleural fluid is not generally found in healthy individuals. Therefore, elevated telomerase levels in pleural fluid would indicate malignancy rather than mere bacterial or viral infection. Finally, the step of quantitatively detecting telomerase activity is not limited to the disclosed protocol. Rather, any mechanism for measuring telomerase levels can be used. For example, reagents or chemical entities that specifically bind to or recognize telomerase, such as telomerase antibodies, may be used. Thus, a variety of known serologic assays, such as complement fixation, ELISA, immunoblots and equivalents thereof , may be used to measure telomerase activity levels.

WHAT IS CLAIMED:

1. A method for detecting cancer in a subject comprising the steps of:

(a) removing the cellular portion of a body fluid specimen collected from said subject;

(b) preparing a protein extract from said body fluid remainder;

(b) assaying said extract for the presence of telomerase RNA or telomerase activity;

(c) quantitating the level of telomerase RNA or telomerase activity; and

(d) comparing the level of telomerase RNA or telomerase activity with the level of telomerase RNA or telomerase activity found in normal levels to determine the presence of cancer.

2. The method of claim 1 wherein said cancer is expressed in the anatomical system selected from the group consisting of the gastrointestinal, endocrine, reproductive, head-neck, liver, breast, renal, urinary, neural, hematological, lymphoid, musculoskeletal, and cardiorespiratory.

3. The method of claim 1 wherein said cancer is lung cancer.

4. The method of claim 1 wherein the cancer is non-metastatic.

5. The method of claim 1 wherein the cancer is metastatic.

6. The method of claim 1 wherein the body fluid is selected from the group consisting of blood, plasma, lymph, pleural fluid, spinal fluid, saliva, sputum, urine, and semen.

7. The method of claim 1 wherein the body fluid is a circulated fluid.

8. The method of claim 1 wherein the body fluid is blood.

9. The method of claim 1 wherein the steps (b) and (c) further comprise the steps of:

(i) incubating the extract with a reaction mixture of telomerase substrate and a buffer so as to catalyze extension of said telomerase substrate by addition of telomeric repeat sequences;

(ii) adding to said mixture a primer comprising a sequence sufficiently complementary to a telomeric repeat to hybridize specifically thereto under conditions such that if an extended telomerase substrate is present, said primer will hybridize to said extended telomerase substrate thereby forming duplex DNA molecules;

10. The method of claim 9 further comprising the step of:

(iii) amplifying duplex DNA to achieve a measurable amount of sample.

11. The method of claim 9 wherein the telomerase substrate is the oligonucleotide TS (5'-AAT CCG TCG AGC AGA GTT-3').

12. The method of claim 9 wherein the primer is CX-ext oligonucleotide (5'-GTG CCC TTA CCC TTA CCC TTA CCC TAA-3').

13. A method for treating a diagnosed cancer in a patient comprising the steps of:

(a) quantitatively measuring telomerase RNA or telomerase activity in body fluid sample withdrawn from the patient to determine an initial telomerase activity level;

(b) treating a patient with a known cancer therapy;

(c) re-measuring the level of telomerase RNA or telomerase activity in the body fluid of the patient after said therapy;

(d) comparing the initial telomerase RNA or telomerase activity levels with the levels measured after said therapy; and

(e) correlating a reduction in telomerase RNA or telomerase activity with the effectiveness of said cancer therapy.

14. The method of claim 10 further comprising repeating steps (b) -(e) over a predetermined period time.

15. The method of claim 13 wherein the body fluid is selected from the group consisting of blood, plasma, lymph, pleural fluid, spinal fluid, saliva, sputum, urine, and semen.

16. The method of claim 13 wherein the body fluid is a circulated fluid.

17. The method of claim 13 wherein the body fluid is blood.

18. A kit for detecting telomerase levels in blood products comprising:

telomerase substrate; a reverse primer comprising a sequence sufficiently complementary to a telomeric repeat to hybridize specifically thereto to form a duplex DNA molecule in the presence of an extended telomerase substrate is present; a reaction buffer, and DNA amplification components.

19. The kit of claim 18 wherein the telomerase substrate comprises TS oligonucleotide (5'-AAT CCG TCG AGC AGA GTT-3') and the reverse primer comprises CX-ext oligonucleotide (5'-GTG CCC TTA CCC TTA CCC TTA CCC TAA-3').

20. The kit of claim 18 wherein the DNA amplification components comprise a high fidelity Taq polymerase.

Figure 1:

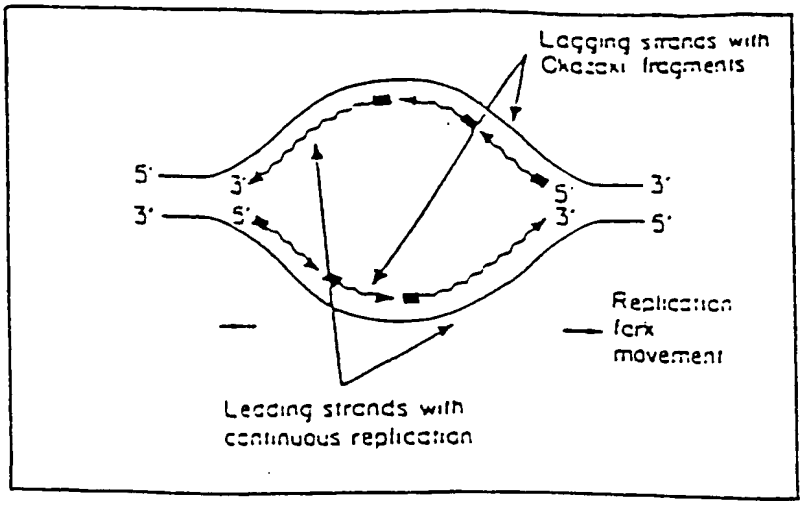


Figure 2:

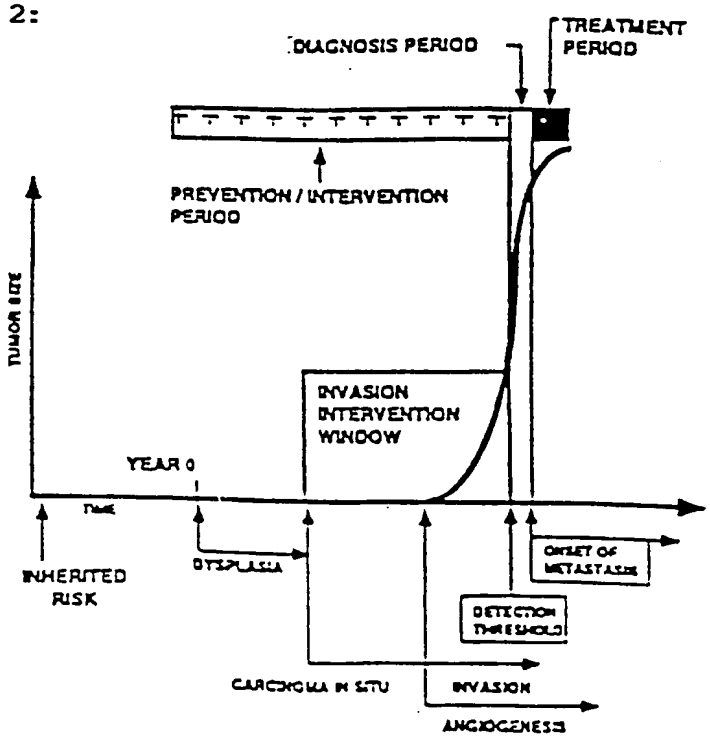


Figure 3:

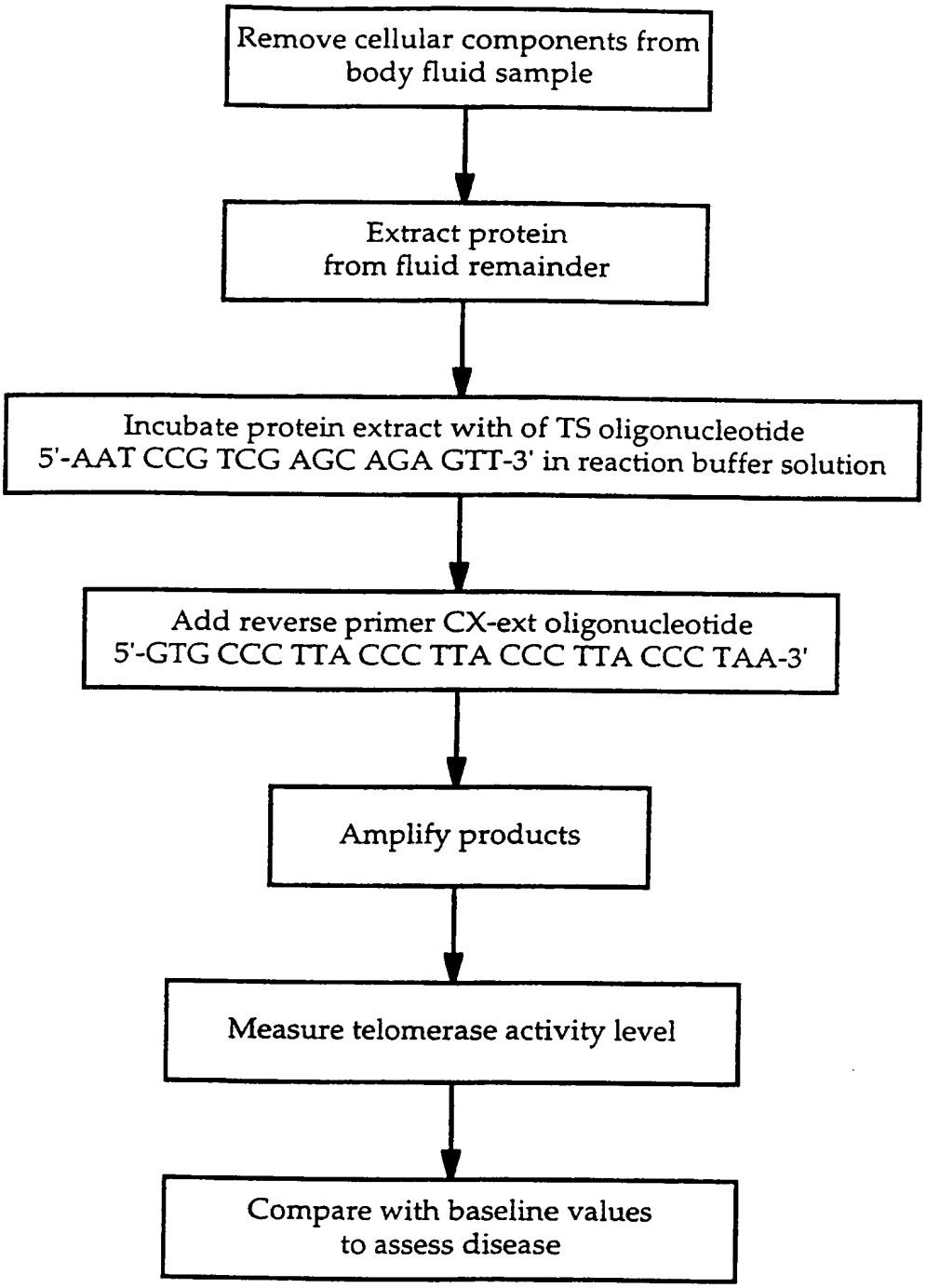

Remove cellular components from body fluid sample
Extract protein from fluid remainder
Incubate protein extract with of TS oligonucleotide 5'-AAT CCG TCG AGC AGA GTT-3' in reaction buffer solution
Add reverse primer CX-ext oligonucleotide 5'-GTG CCC TTA CCC TTA CCC TTA CCC TAA-3'
Amplify products
Measure telomerase activity level
Compare with baseline values to assess disease

Figure 4:

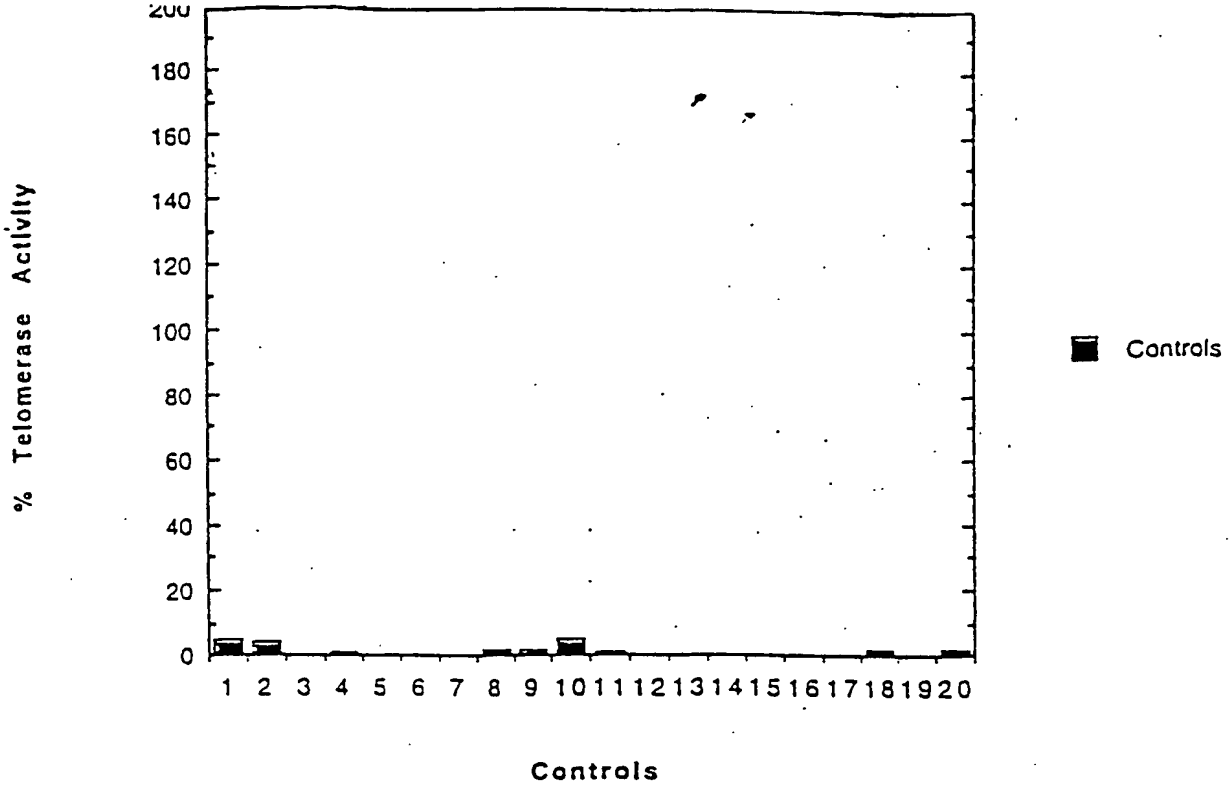
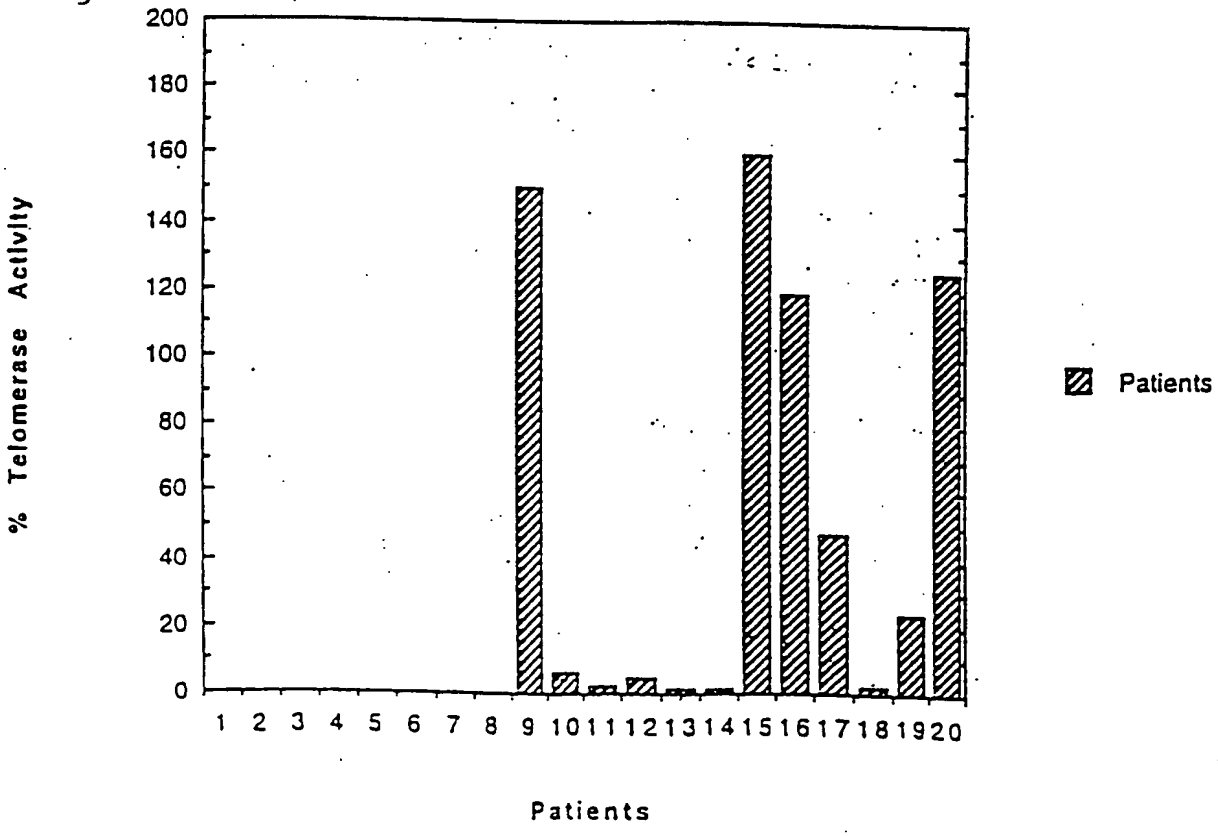


Figure 5:



SEQUENCE LISTING

<110> Strovel, Jeffrey W
Stamberg, Judith
Abruzzo, Lynne V
Highsmith, Edward

<120> Telomerase Assay of Body Fluids for Cancer Screening and Assessment of Disease Stage and Prognosis

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03302

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/00, 1/68; C12P 19/34
US CL :435/4, 6, 7.4, 91.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 6, 7.4, 91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P --- X,P	US 5,830,644 A (WEST et al.) 03 November 1998, especially columns 42-74.	1-17 --- 18-20
A,P --- X, P	US 5,840,490 A (BACCHETTI et al.) 24 November 1998, see entire document.	1-17 --- 18-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 APRIL 1999	Date of mailing of the international search report 05 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer IREM YUCEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03302

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN(CAPLUS), DIALOG(MEDLINE, BIOSIS, SCISEARCH, CANCERLIT, PASCAL)
Terms: Inventors' names, TRAP, telomerase, activity, assay?, screen? diagnos? secret? export?, blood, body or biological fluid?, PCR, detect?amplif?, protein? extract?, tumor?, cancer?